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Roles of *Drosophila* DJ-1 in Survival of Dopaminergic Neurons and Oxidative Stress

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Summary

The loss of dopaminergic neurons in the substantia nigra is the pathological hallmark of Parkinson's disease (PD). While the etiology of sporadic PD remains elusive, an inherited form of early-onset familial PD is linked to mutations of DJ-1 [1]. To understand the biological function of DJ-1 and its relevance to the pathogenesis of PD, we investigated the function of DJ-1 using *Drosophila*. *Drosophila* possesses two homologs of human DJ-1: DJ-1α and DJ-1β. We found that *DJ-1α* is expressed predominantly in the testis, while *DJ-1β* is ubiquitously present in most tissues, resembling the expression pattern of human DJ-1. Loss-of-function *DJ-1β* mutants demonstrated an extended survival of dopaminergic neurons and resistance to paraquat stress, but showed acute sensitivity to hydrogen peroxide treatment. We showed a compensatory upregulation of *DJ-1α* expression in the brain of the *DJ-1β* mutant and demonstrated that overexpression of *DJ-1α* in dopaminergic neurons is sufficient to confer protection against paraquat insult. These results suggest that *Drosophila* homologs of DJ-1 play critical roles in the survival of dopaminergic neurons and response to oxidative stress.

Results and Discussion

DJ-1α and *DJ-1β* Show Distinct Temporal and Spatial Expression Patterns

Two *Drosophila* genes, *DJ-1β* (*CG1349*) and *CG6646*, share significant homology with human DJ-1 (both show 69% similarity to human; see Figure S1 in the Supplemental Data available with this article online). We will subsequently refer to *CG6646* as *DJ-1α*. The existence of multiple *Drosophila* homologs for a single human gene is rare, and therefore we sought to establish the relationship between *DJ-1α* and *DJ-1β* in terms of their expression. Developmental expression patterns for both genes were determined by quantitative real-time PCR. RNA samples were collected from defined developmental stages: 2 hr embryos (at which time only maternally contributed RNA is present), 2–16 hr embryos, 1–3 instar larvae, late pupae, and adult flies (10 days old). The two genes showed distinct developmental expression (Figures 1A and 1B). *DJ-1α* was expressed at high levels only in the later stages of development, i.e., pupae and adults. *DJ-1β* did not show such large changes in expression levels; it is maternally contributed, after which its levels fall slightly in the embryo but increase again through development. We also established the spatial expression of *DJ-1α* and *β* in adults. Comparing *DJ-1α* expression levels in male and female heads and bodies showed that *DJ-1α* mRNA expression was greatly increased in male bodies, strongly suggesting localization of the *DJ-1α* transcript in the testis. Fly bodies were therefore dissected to remove the reproductive organs (either testis or ovary). *DJ-1α* transcripts were presented mainly in the testis (Figure 1E). However, *DJ-1β* showed relatively constant expression levels throughout heads and bodies (Figure 1D).

Loss of *DJ-1β* Expression Results in Increased Survival of Dopaminergic Neurons

Since the expression pattern of *Drosophila DJ-1β* resembles human DJ-1, which is widely expressed in most tissues [1], we generated mutant flies lacking expression of DJ-1β. Using P element local hopping from the EP line, EP3700, which contains a single P element insertion located 700 bp downstream of *DJ-1β,* we isolated two mutant lines in which DJ-1β expression was disrupted due to the insertion of the P element into the gene. Loss of protein expression was confirmed by Western blotting using an antibody generated to a peptide region of DJ-1β that shows no homology to DJ-1α (Figures 1F and 1G).

As the major pathological hallmark of PD is the loss of dopaminergic neurons from the substantia nigra, we investigated the effect of loss of *DJ-1β* expression on dopaminergic neurons in *Drosophila*. Total dopaminergic neuron number was counted in whole brains following staining with an antibody to tyrosine hydroxylase (TH), an enzyme in the dopamine synthesis pathway, which is a marker for dopaminergic neurons (Figure 2). In *DJ-1β* mutant flies (aged 20 or 40 days at 25°C), no difference was seen in the total number of dopaminergic neurons compared with control. This is in contrast to transgenic flies overexpressing human α-synuclein where a specific loss of dorsomedial dopaminergic neurons was seen as early as 10 days of age [2, 3]. An age-related decrease in the number of TH-positive neurons was seen in normal flies as previously reported [4]. The intensity of immunoreactivity against TH of the remaining dopaminergic neurons was also reduced (Figure 2). Surprisingly, we found that dopaminergic neurons were mostly sustained in the older *DJ-1β* mutant flies, suggesting that the absence of DJ-1β extended the survival of the dopaminergic neurons. Immunoreactivity of these cells was also decreased to a lesser extent.

Since flies overexpressing human α-synuclein showed selective regional loss of dopaminergic neurons [2, 3], we examined whether the *DJ-1β* mutants also show protection of dopaminergic cells in specific

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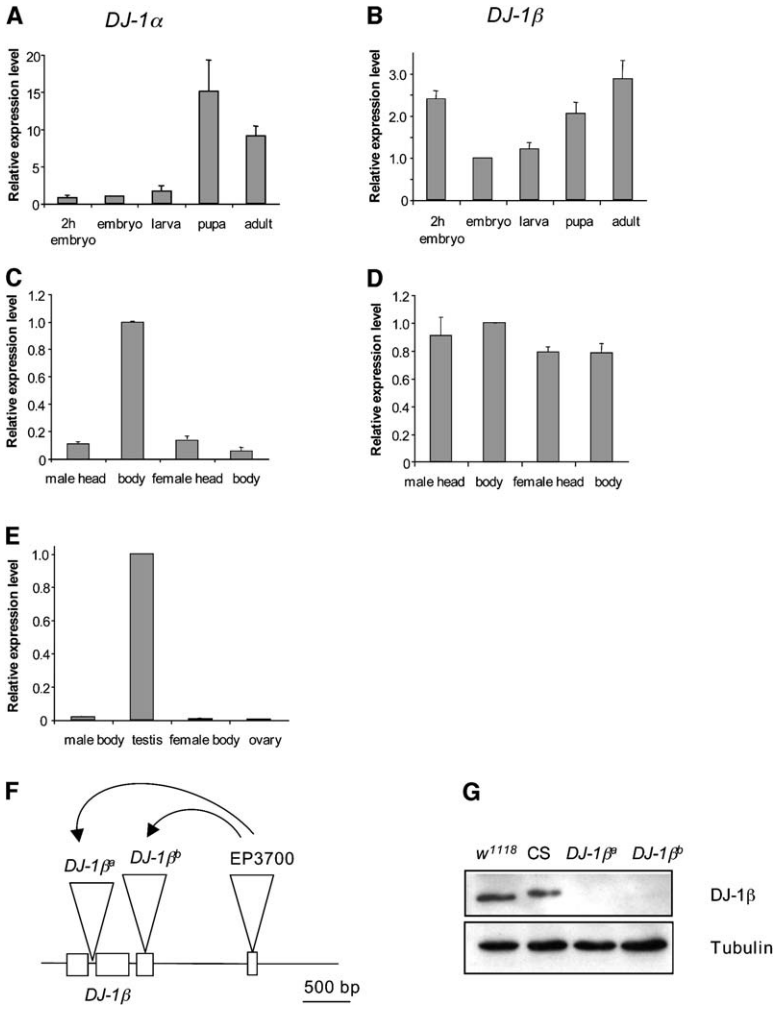

Figure 1. Expression Pattern of *Drosophila* *DJ-1α* and *DJ-1β* and Generation of the *DJ-1β* Mutant

(A) *DJ-1α* mRNA transcript is most abundant in later developmental stages.

(B) *DJ-1β* mRNA is maternally contributed and shows less variation throughout development.

(C) *DJ-1α* is present at highest levels in male bodies.

(D) *DJ-1β* is present in head and body tissue at similar levels.

(E) *DJ-1α* is greatly concentrated in the testis. Expression levels are shown as fold change relative to expression in embryos (A, B), male body (C, D), and testis (E). Quantitative RT-PCR results were obtained from cDNA isolated from defined developmental stages, and values were normalized to the level of *Actin5C*, which remain constant throughout development. Values represent mean ± SEM. Three independent experiments were done in triplicate.

(F) Two alleles of the *DJ-1β* mutants were isolated using P element local hopping. A P element is inserted in *DJ-1β^a^* at base pair 178 and in *DJ-1β^b^* at base pair 739 of the *DJ-1β* gene.

(G) Western blot analysis shows no *DJ-1β* protein in the *DJ-1β* mutants. Tubulin was used as loading control.

areas of the brain. We found that dopaminergic cell clusters in PPL1, PPM1/2, and PPL2 regions survived better compared to other regions (Figure 3).

To determine if this protective effect is universal or selective for dopaminergic neurons, we investigated survival of serotoninergic neurons (Figure 3D). The number of serotoninergic neurons in normal and *DJ-1β* mutant flies at 60 days of age was not changed compared to that of 10-day-old flies, although immunoreactivity against serotonin was reduced in both flies. This suggests that the effect of DJ-1β loss is not general, but specific to dopaminergic neurons.

Next, we investigated whether the protection of these neurons improved motor function in the mutant flies using negative geotaxis assays. Although 60-day-old mutant flies have more TH-positive neurons, they displayed sedentary behavior similar to normal flies of the same age (data not shown). This implies that extended survival of specific dopaminergic neurons does not influence locomotor vigor in old flies.

Loss of DJ-1β Expression Protects Flies against Paraquat Insult

A role for DJ-1 in oxidative stress response has been proposed using cell-culture systems [5–8]. We investigated the effect of paraquat stress on *DJ-1β* mutant flies. Flies raised for 5 days from eclosion at 25°C were administered 20 mM paraquat in 5% sucrose (Figure 4A). Notably, the mutant flies showed significant resistance to paraquat treatment, while control flies exhibited acute sensitivity to paraquat, suggesting that the *DJ-1β* mutants are less vulnerable to oxidative insult. To ensure that changes in sensitivity to paraquat did not result from altered feeding behavior, we measured the level of radiolabeled sucrose consumption in flies used in the oxidative stress test, and no changes in consumption were found (Figure S2).

Decreased Sensitivity to Paraquat in the *DJ-1β* Mutants Is Due to a Compensatory Upregulation in *DJ-1α* in the Brain

Given that DJ-1β shares significant homology with DJ-1α, it is plausible that DJ-1α may play a role in the phenotypes found in the *DJ-1β* mutant. We therefore investigated the possibility of a compensatory upregulation of *DJ-1α* in the *DJ-1β* mutant. The levels of *DJ-1α* mRNA transcripts, as measured by quantitative real-time PCR, were doubled in the mutant brains (Figure 4B), but not in the whole flies (data not shown). To confirm whether this upregulation of *DJ-1α* is indeed rele-

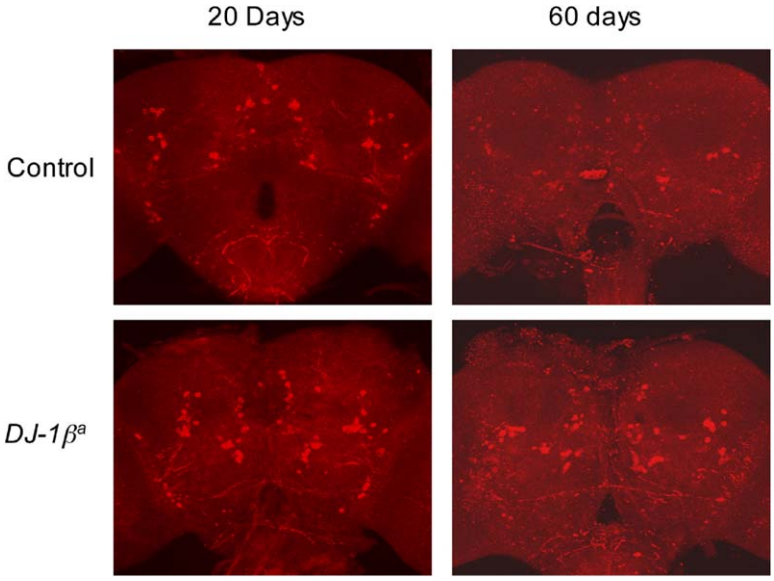

Figure 2. Tyrosine Hydroxylase Immunostaining of the Fly Brain

Tyrosine hydroxylase (TH) immunostaining was used to identify dopaminergic neurons (DA) in the brain. Individual dompaminergic neurons can be clearly seen in whole-mount *Drosophila* brains. Immunoreactivity to TH is decreased between 20 days and 60 days of age in control flies, but not in the *DJ-1β* mutant. Images shown were collected by confocal microscopy using a Zeiss LSM 520 microscope.

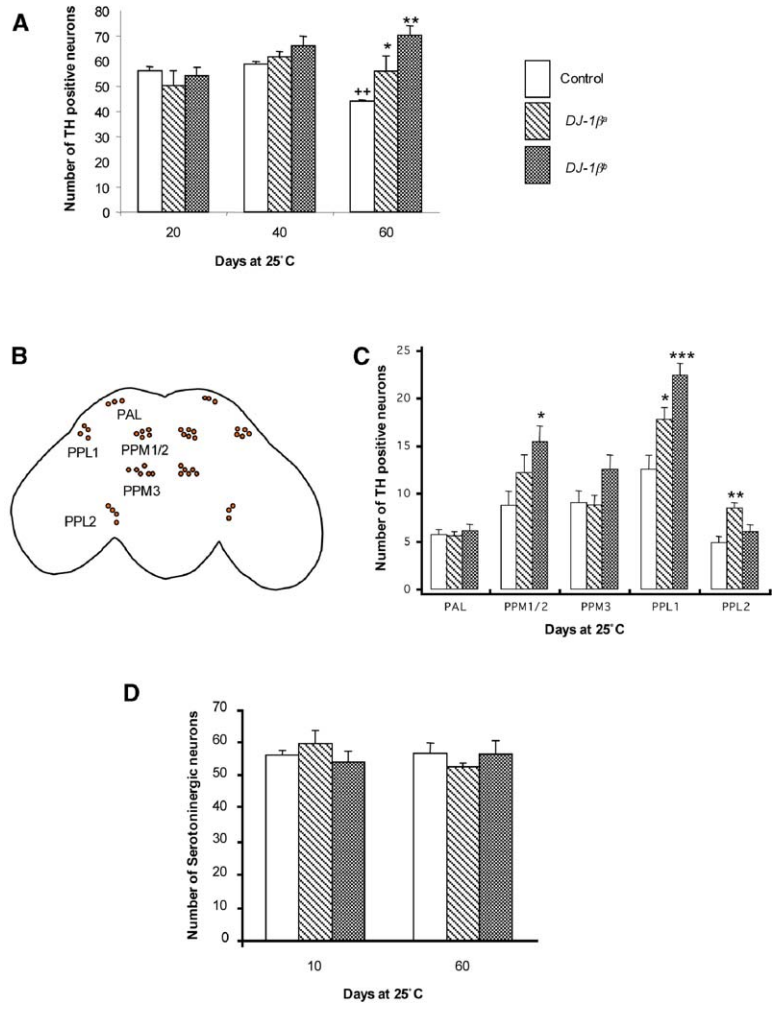

Figure 3. The Number of DA in the *DJ-1β* Mutant Is Not Reduced as the Fly Ages

(A) DA was counted in whole-mount *Drosophila* brains. Total number of TH-positive neurons is reduced at 60 days in control flies ($^{2+}$ p = 0.0028, 40-day-old versus 60-day-old). The *DJ-1β* mutant shows survival of DA neurons and little change in immunoreactivity (*p = 0.01, **p = 0.0009 versus control 60-day-old).

(B) DA can be classified by their localization in readily identifiable groups.

(C) Cells in PPL1, PPM1/2, and PPL2 clusters show extended survival in the *DJ-1β* mutant (*p < 0.05; **p < 0.01; ***p < 0.001).

(D) Staining of whole-mount brains with antiserotonin antibodies reveals no age-dependant decrease in serotoninergic neurons (SN) in the brains of control and the *DJ-1β* mutant. Six brains were used for DA or SN counting at each stage. Values represent mean ± SEM.

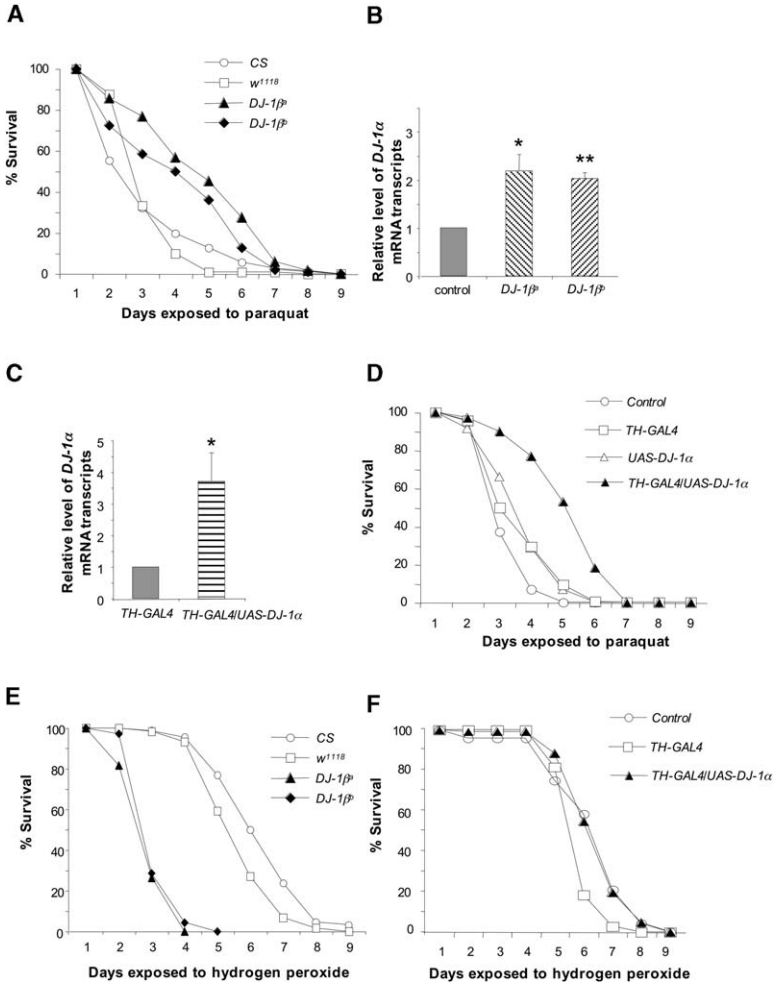

Figure 4. Sensitivity of *Drosophila DJ-1* Mutants to Oxidative Stress

(A) The *DJ-1β* mutants are resistant to paraquat insult ($p < 1 \times 10^{-4}$ for either *DJ-1β* mutant versus either control).

(B) The *DJ-1α* mRNA transcript levels are doubled in the brain of *DJ-1β* mutants (*p = 0.027, **p = 0.001).

(C) *DJ-1α* is overexpressed in dopaminergic neurons using the *TH-GAL4* driver (*p = 0.0256).

(D) Overexpression of *DJ-1α* in dopaminergic neurons is sufficient to protect flies against paraquat treatment ($p < 1 \times 10^{-4}$ for flies overexpressing *DJ-1α* versus each control).

(E) The *DJ-1β* mutants exhibit acute sensitivity to 1% H_2O_2 treatment ($p < 1 \times 10^{-4}$ for *DJ-1β*^{a} or *DJ-1β*^{b} versus each control).

(F) Overexpression of *DJ-1α* in dopaminergic neurons is not sufficient to protect flies against H_2O_2 treatment. Quantitative RT-PCR was repeated three times in triplicate, and statistical analysis was carried out using Student's t test. Values represent mean ± SEM. Oxidative stress tests were performed at least three times with an average of 80 male flies each time, and statistical analysis was performed using Kaplan-Meier analysis with log rank post-test.

vant to the altered response of the *DJ-1β* mutant to oxidative stress, particularly in dopaminergic neurons, we created transgenic flies overexpressing *DJ-1α* in dopaminergic neurons using the binary UAS/GAL4 system. Flies containing the *DJ-1α* transgene were crossed with the *TH-GAL4* (TH) driver lines [9] to yield *DJ-1α* overexpression specifically in dopaminergic neurons. Figure 4C shows that the *DJ-1α* transcript level was elevated about 3.5-fold in the brain of transgenic flies containing the driver. We then measured the survival of transgenic flies following paraquat treatment (Figure 4D). *TH-GAL4/UAS-DJ-1α* flies showed significant resistance to paraquat toxicity similar to that of the *DJ-1β* mutants, suggesting that overexpression of *DJ-1α* in dopaminergic neurons alone can preserve the animal against oxidative stress.

DJ-1β Mutant Flies Show an Increase in Sensitivity to Hydrogen Peroxide Stress

To investigate whether *DJ-1β* mutant flies are also insensitive to other oxidative stress condition, we examined the effect of hydrogen peroxide on these flies. Flies were treated with H_2O_2 and their survival determined as for paraquat exposure. The *DJ-1β* mutant flies were extremely sensitive to H_2O_2 (Figure 4E). These data are consistent with cell-culture experiments demonstrating DJ-1-deficient cells to be highly sensitive to oxidative stress [6]. We also measured the survival of *TH-GAL4/UAS-DJ-1α* flies against H_2O_2 treatment and found no effect of *DJ-1α* overexpression (Figure 4F). These results suggest that DJ-1β plays a critical role in H_2O_2-induced oxidative stress response.

Notably, the *DJ-1β* mutant flies exhibit difference in response to different forms of oxidative stress. The flies show less sensitivity to paraquat but increased sensitivity to H_2O_2. The upregulation of *DJ-1α* is therefore insufficient to protect against H_2O_2, unlike paraquat. This suggests that DJ-1α is crucial for protecting cells against paraquat-mediated stress, while DJ-1β plays a role in responding to H_2O_2-induced stress. The exact mechanism by which paraquat results in the production of reactive oxygen species is unknown, although the major species produced is superoxide. The two homologs of DJ-1 in *Drosophila* may therefore be required to protect against different species of reactive oxygen. Evidence is also emerging from mouse models to support the possibility of selective response of DJ-1 to oxidative stress as DJ-1-deficient mice demonstrate an increased sensitivity to MPTP [10], but no such increase is seen in response to paraquat [11]. These results may

provide new insights into the mechanism associated with DJ-1 in PD.

While mammalian DJ-1 apparently plays multiple roles, such as sensing oxidative levels, chaperone activity, and fertility, in *Drosophila* these roles may be divided between the two homologs. It remains to be determined whether DJ-1β is required for the maintenance of dopaminergic neurons in the absence of DJ-1α upregulation and whether DJ-1α has a role in dopaminergic neurons under normal circumstances. In normal fly brains, the level of *DJ-1α* transcripts appeared to be low, perhaps suggesting that the major role of DJ-1α is in the testis. However, it is also possible that DJ-1α is expressed at significant levels in specific regions of the brain where it may also have an important function.

Further Discussion

An accompanying paper by Meulener et al. in this issue [12] shows that mutant flies lacking both *DJ-1α* and *β* are sensitive to paraquat stress, while the *DJ-1β* mutants in this report are resistant to the stress. This may offer an interesting insight into the nature of the interaction between the DJ-1 homologs. The absence of a decrease in sensitivity to paraquat in the *DJ-1β* mutants created by Meulener et al. suggests that no compensatory upregulation in *DJ-1α* occurs in these flies, in contrast to that seen in our mutant lines. A major difference between the mutant lines described is that those of Meulener et al. are created by a deletion, which also removes adjacent genomic DNA regions that are still present in the mutants generated in our lab. Within these DNA regions, it is likely that regulatory elements exist. A recent report by Spilianakis et al. [13] showed that regulatory regions of related genes on different chromosomes are important in the coordinate regulation of multiple genes. It is therefore possible that the two *Drosophila* DJ-1 homologs are also coordinately regulated. A second possibility for the variation between the two mutant lines may be that the P element insertion lines created in our lab could express a truncated version of DJ-1β. No evidence of such a product was observed by Western blotting; however, the antibody generated for this study recognizes a region close to the C terminus of DJ-1β, so its existence cannot be ruled out. It is possible that a truncated protein product could result in the compensatory upregulation of the *DJ-1α* gene we observe. Further work will help to elucidate the exact role of DJ-1 homologs in paraquat resistance.

Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/17/1578/DC1/.

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